The Basics: What is a Nuclease Protection Assay?

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What is a Nuclease Protection Assay?

Nuclease protection assays (NPAs), including both ribonuclease protection assays (RPAs) and S1 nuclease assays, are an extremely sensitive method for the detection, quantitation and mapping of specific RNAs in a complex mixture of total cellular RNA. The basis of NPAs is a solution hybridization of a single-stranded, discrete sized antisense probe(s) to an RNA sample (see Figure 1). The small volume solution hybridization is far more efficient than more common membrane-based hybridization, and can accommodate up to 100 µg of total or poly(A) RNA. After hybridization, any remaining unhybridized probe and sample RNA are removed by digestion with a mixture of nucleases. Then, in a single step reaction, the nucleases are inactivated and the remaining probe:target hybrids are precipitated. These products are separated on a denaturing polyacrylamide gel and are visualized by autoradiography. If nonisotopic probes are used, samples are visualized by transferring the gel to a membrane and performing secondary detection.

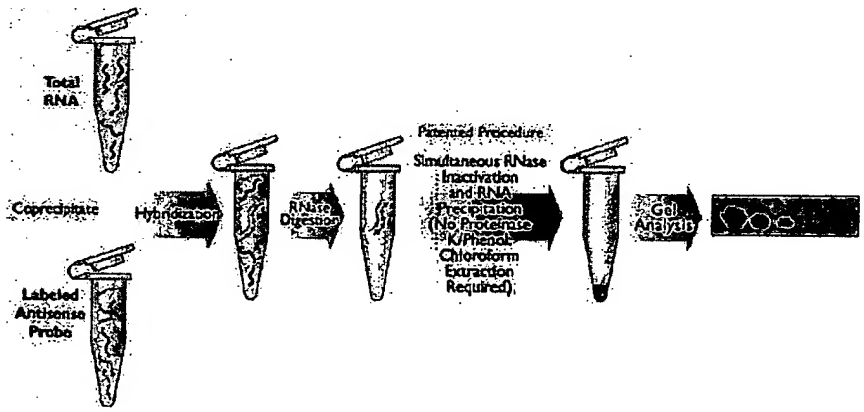

Figure 1. Detection of Specific mRNA Species Using a Nuclease Protection Assay.

NPAs are the method of choice for the simultaneous detection of several RNA species. During solution hybridization and subsequent analysis, individual probe/target interactions are completely independent of one another. Thus, several RNA targets and internal controls can be assayed simultaneously (up to twelve have been used in the same reaction), provided that the protected

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fragment of individual probes are of different lengths. NPAs are also commonly used to precisely map mRNA termini and intron/exon junctions.

RNA Quantitation

To quantitate mRNA levels using NPAs, the intensities of probe fragments protected by the sample RNA are compared to the intensities generated from either an endogenous internal control (relative quantitation) or known amounts of sense strand RNA (absolute quantitation). For more information on using NPAs for quantitation, see Technical Bulletin 151, "**Use of Internal and External Standards or Reference RNAs for Accurate Quantitation of RNA Levels.**"

Advantages of NPAs over Northerns

- NPAs are more sensitive than traditional Northerns. They can be used to detect as little as 5 femtograms of target RNA or 4,000 to 50,000 copies/sample.
- NPAs are more tolerant of partially degraded RNA than Northerns. If samples are even slightly degraded, the quality of data from a Northern blot is severely compromised.
- NPAs are able to distinguish between transcripts of multi-gene families that may comigrate on Northerns.
- NPAs can be used to map mRNA termini and intron/exon junctions.
- Multi-probe assays are easy to perform with NPAs (see Figure 2). Up to 10 probes plus one or two internal controls can routinely be analyzed from a single RNA sample using Ambion's **RPA III™ Kit**. While multi-probe analysis is possible with Northern analysis it is very time consuming and requires multiple stripping and reprobing of a single blot.

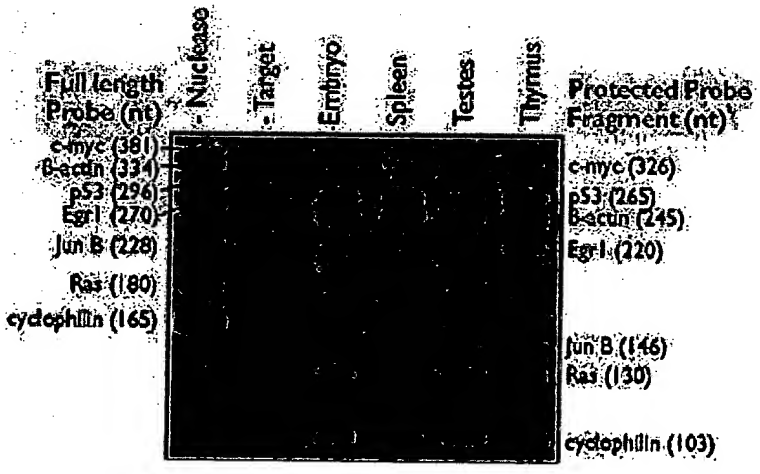

Figure 2. Simultaneous Quantitation of Multiple mRNAs Using the RPA III® Kit. Ten micrograms of various mouse tissue total RNAs were hybridized overnight with approximately 50,000 cpm each of seven distinct probe transcripts. Nuclease digestion, product separation on a denaturing 6% acrylamide gel, and a four-hour exposure to film at -80°C were used to assess hybridization levels. The radiolabeled probes were synthesized in 5 µl transcription reactions using Ambion's **MAXIscript™**

Kit with [α-^{32}P]UTP (800 Ci/mmol, 10 Ci/ml) and gel purified prior to hybridization. The specific activities of the cyclophilin and ß-actin probes were reduced twenty and 200-fold, respectively, by adding appropriate amounts of non-radioactive UTP to the transcription reaction.

Advantages of NPAs over RT-PCR Reactions

- RPAs are easy to use and don't require extensive optimization. Optimization reactions must be preformed prior to each RT-PCR reaction and the data obtained by RT-PCR analysis can be confusing and difficult to interpret.
- RPAs don't require expensive equipment purchases.
- Multi-probe assays are easy to perform with RPAs (see Figure 2). Up to 10 probes plus one or two internal controls can routinely be analyzed from a single RNA sample using Ambion's **RPA III™ Kit**. Due to interactions between primers, it is difficult to multiplex with greater than two primer sets using RT-PCR analysis.
- RPAs are able to distinguish between transcripts of multi-gene families that are not currently characterized.

Limitations of Nuclease Protection Assays

The primary limitation of NPAs is the lack of information on transcript size. The portion of probe homologous to target RNA determines the size of the protected fragment. Another drawback to NPAs is the lack of probe flexibility. The most common type of NPA, the ribonuclease protection assay, requires the use of RNA probes. Oligonucleotides and other single-stranded DNA probes can only be used in assays containing S1 nuclease. A region of the single-stranded, antisense probe must typically be completely homologous to target RNA to prevent cleavage of the probe:target hybrid by nuclease. This means that partially related sequences (e.g., probe and target RNA from different species) usually cannot be used.

Steps Involved in Nuclease Protection Assays

RNA Isolation

There are a number of protocols, techniques and commercially available kits that can be used to isolate RNA for NPAs, i.e., most if not all RNA isolation methods are compatible with NPAs. RNA isolation techniques all share these common attributes:

- Cellular lysis and membrane disruption
- Inhibition of ribonuclease activity
- Deproteinization
- Recovery of intact RNA

Ambion provides several options for isolation of total RNA and mRNA that are compatible with a variety of cells and tissues, including bacteria, yeast, plant and animal. For a further discussion of RNA isolation options, see **RNA Isolation: The Basics**.

Probe Generation and Purification

The type of probe used (RNA vs. DNA) is dependent upon which nuclease is used in the digestion step. Ribonuclease protection assays (i.e., **RPA III™**, **RPA II™**, **HybSpeed™ RPA**, and **Direct Protect™ Lysate RPA** Kits) all require the use of RNA probes.

It is essential that probes used in NPA analysis are all of a discrete length. High specific activity single-stranded RNA probes can be produced by in vitro transcription reactions using Ambion's **MAXIscript™ Kit**. To assure that the probe is full length we recommend gel purification. Ambion's **Technical Bulletin 171** discusses gel purification of probes in detail.

Either radiolabeled or nonisotopically labeled probes can be used for NPA analysis. We recommend that nonisotopic nucleotides be incorporated enzymatically (versus post synthesis chemical labeling of probes). Technical Bulletin 173, "**Methods for Nonisotopic Labeling**," describes how the MAXIscript Kit can be used to incorporate nonisotopic nucleotides into RNA probes.

For accurate quantitation of a specific message, probe concentration must be in molar excess over the target mRNA. This necessitates the use of low specific activity probes for abundant targets. For moderately abundant messages (e.g., ß-actin, GAPDH, or cyclophilin), a 1:50 dilution of labeled NTP with "cold" NTP should be used to increase the molar amount of probe made while simultaneously reducing the specific activity of the probe. For very abundant messages (e.g., 18S rRNA or 28S rRNA), a 1:10,000 dilution with "cold" NTP should be used.

NPA Analysis

Ambion's NPA kits provide many advantages over traditional and homebrewed NPAs. These advantages include:

- **Ease of use.** A patented technology is used which allows for a single-tube reaction with no phenol extraction or proteinase K digestion.
- **Increased sensitivity.** Ambion's **RPA III™ Kit** is the most sensitive RPA available.
- **Faster reactions.** Ambion's **HybSpeed™ RPA Kit** has a revolutionary 10-minute hybridization step allowing an RPA reaction to be completed in a single day.

For more information to help you choose the kit which is ideal for your needs, read "**Which Nuclease Protection Assay Kit Is Right for Me?**"

Denaturing Acrylamide Gel Electrophoresis

The number and size of probes will dictate the gel size and acrylamide concentration. Typically a 5% denaturing acrylamide gel is used. This will

effectively resolve fragments of about 500–1000 nucleotides. It is useful to have size markers on the gel. Single-stranded RNA markers are the most accurate (e.g., Ambion's **RNA Century Markers** or **Century Marker Templates**), but double-stranded DNA markers can be used if it is not critical to know the exact size of the products.

Ordering Information
For prices and availability, please contact **Customer Service**.

Cat#	Product Name	Size
1308	MAXIscript® SP6 Kit	30 rxns
1310	MAXIscript® SP6 Kit	100 rxns
1312	MAXIscript® T7 Kit	30 rxns
1314	MAXIscript® T7 Kit	100 rxns
1316	MAXIscript® T3 Kit	30 rxns
1318	MAXIscript® T3 Kit	100 rxns
1320	MAXIscript® SP6/T7 Kit	15 rxns each
1322	MAXIscript® SP6/T7 Kit	50 rxns each
1324	MAXIscript® T7/T3 Kit	15 rxns each
1326	MAXIscript® T7/T3 Kit	50 rxns each
1410	RPA II™	120 rxns
1412	HybSpeed™ RPA Kit	100 rxns
1414	RPA III™ Ribonuclease Protection Assay Kit	100 rxns
1415	RPA III™ Ribonuclease Protection Assay Kit	35 rxns
1420	Direct Protect™ Lysate RPA Kit	100 rxns
7140	RNA Century™ Markers	50 µg
7145	RNA Century™-Plus Markers	50 µg
7175	BrightStar® Biotinylated RNA Century™ Markers	50 lanes
7180	BrightStar® Biotinylated RNA Century™-Plus Markers	50 lanes
7780	Century™ Marker Templates	5 µg
7782	Century™-Plus Marker Templates	5 µg

A. Background: The Ribonuclease Pr tection Assay

The Ribonuclease Protection Assay (RPA) is an extremely sensitive procedure for the detection and quantitation of RNA species (usually mRNA) in a complex sample mixture of total or Poly(A) selected RNA. For the RPA, a labeled (nonisotopic or radioactive) RNA probe is synthesized that is complementary to part of the target RNA to be analyzed. This is done by placing the 3' end of the probe sequence adjacent to one of the phage polymerase promoters (T3, T7, or SP6) by cloning into a plasmid vector or by using a PCR primer that contains the promoter sequence. The corresponding T3, T7, or SP6 RNA polymerase is then used to generate an antisense RNA transcript by ***in vitro*** transcription. The labeled probe and sample RNA are incubated under conditions that favor hybridization of complementary sequences. After hybridization, the mixture is treated with ribonuclease to degrade unhybridized probe. Labeled probe that is hybridized to complementary RNA from the sample will be protected from ribonuclease digestion, and can be separated on a polyacrylamide gel and visualized either by autoradiography (radioactively-labeled probes) or by a secondary detection procedure (nonisotopically-labeled probes). When the probe is present in molar excess over the target in the hybridization reaction, the intensity of the protected fragment will be directly proportional to the amount of target RNA in the sample mixture. Ribonuclease protection assays are thus analogous to S1 nuclease protection assays, but ribonuclease is generally acknowledged to be easier to fine-tune and less prone to degrade double-stranded nucleic acid than S1 nuclease (Molecular Cloning, 1989; and Friedberg, 1990).

High-speed Hybridization

A major limitation in hybridization-based assays is that the hybridization step typically requires overnight or longer incubations; this is particularly true when the target molecule is present at very low levels (sub-picogram). Several approaches have been found to speed up nucleic acid hybridization reactions. One approach is to increase the probe concentration. However, this can result in unacceptably high levels of background signal (noise). The addition of neutral dextran polymers are known to increase rates about 10-fold (Wetmur, 1968), and dextran sulfate can increase apparent hybridization rates as much as 100-fold for Southern blots (Wahle et al., 1979). This increase can be attributed to the "volume exclusion principle", in which the concentration of the nucleic acid molecules is increased by reduction in the effective solvent volume as a consequence of hydration of the polymers. However, this approach also often leads to increased levels of background signal. Research at Ambion has lead to the development of the HybSpeed RPA kit which allows the hybridization step, normally requiring 12-24 hours, to go to completion in 10 minutes or less without the use of volume excluding polymers (patent pending). Figure 1 demonstrates the kinetics of the HybSpeed hybridization reaction. An additional benefit is that the HybSpeed RPA is 1.5 to 3 times more sensitive than the RPA II, and frequently yields sharper bands. This is presumably due to the increased stability of RNA duplexes in the hybridization buffer and the resulting reduction in nonspecific cleavage at RNase hypersensitive sites. The hybridization using the HybSpeed buffer is carried out at 68°C. This elevated temperature aids in reducing secondary structure of the mRNA and probe, resulting in less background due to undigested self-protected probe. Ambion's HybSpeed RPA kit combines the convenience of a single-tube assay (developed with the RPA II Kit) with the HybSpeed Technology to reduce the overnight hybridization time to ten minutes. The reduction in hybridization time will often allow the complete protection assay to often be performed in one day. The protected probe fragments are then analyzed using PAGE and autoradiography, phosphor imaging, direct imaging, or may be transferred to nitrocellulose or nylon membranes and detected using protocols recommended for the particular type of non-isotopically labeled probe used (Turnbow and Garner, 1993).

The HybSpeed RPA also allows multiprobe analysis within a single RNA sample, so that samples can be simultaneously hybridized with experimental probes and internal standards. Finally, the HybSpeed RPA includes a control vector for mouse β-actin and total RNA from mouse liver. These reagents allow researchers to self-test the quality of the kit and its protocol at any time.

Figure 1. Kinetics of Hybridization for Ambion's HybSpeed™ Technology.

Ten µgs of total mouse liver RNA probed with a 360 base antisense in vitro transcript for mouse β-actin. The coprecipitated RNA and probe were heat denatured in HybSpeed™ hybridization buffer and allowed to hybridize at 68°C for the indicated times. Digestion buffer was added to degrade unhybridized probe. The 250 bp RNA duplex was recovered and run on a 5% polyacrylamide/8M Urea/1X TBE denaturing gel and analyzed by autoradiography. The standard RPA II™ sample was hybridized at 42°C overnight in RPA II™ hybridization buffer.

B. Hybridization of Probe and Sample RNA

NOTE:
Samples with less than 50 µg of RNA; should be normalized to 50 µg with the yeast RNA provided. We generally recommend using gel purified probe or probe that has been determined to consist mainly of full-length transcript as assessed by gel electrophoresis, see Section V.A.

1. For each experimental tube, mix labeled probe (approximately 100-800 pg of 250 nt or 1-10 fmol or 2 - 8 x 10^4 cpm high specific activity) with sample RNA (<1-20 µg) in a ***0.5 ml microfuge tube*** Set up 2 control tubes for every probe to be used, by mixing 10 µl Yeast RNA (50 µg yeast total RNA) with the same amount of labeled probe used for the experimental tubes.
2. Co-ethanol precipitate the probe and sample RNAs by adjusting the final NH_4OAc concentration to 0.5 M and adding 2.5 volumes of EtOH and mixing thoroughly. 5 M NH_4OAc is supplied with the kit for this purpose.
3. Place tubes in -20°C freezer for 15 minutes.
4. Pellet probe and sample RNA for 15 minutes at maximum speed of microcentrifuge, preferably at 4°C.
5. Carefully remove EtOH supernatant from each tube; allow samples to air dry for 5 minutes at room temperature.
6. To each pellet add 10 µl of HybSpeed Hybridization Buffer (pre-heated to 95°C) and immediately put the tube in a 95°C heating block or water bath. After all samples have HybSpeed Hybridization Buffer, thoroughly vortex each sample for 15 sec. and return them to the 95°-100°C bath. Repeat this vortexing Step 2-4 times, until all the pellets are completely dissolved. Do not be concerned about the foaming that may occur. Take care to keep the samples as close to 95°C as possible while resuspending the pellets, because RNA is not soluble in the HybSpeed Hybridization Buffer at temperatures below about 65°C. Complete solubilization of the co-precipitated probe + RNA is essential for maximizing the sensitivity of the HybSpeed System.
7. Heat all tubes at 95°C±5°C for 2-3 minutes after the last sample is resuspended.
8. Incubate tubes in a 68°C incubator (in a preheated tube rack) or submerge in 68°C water bath or heat block for 10 min. to permit hybridization of the probe and complementary mRNA in the sample RNA. Do not allow the samples to drop below 68°C. Transfer from the 95°C bath to the 68°C

incubation should take no more than 30 seconds.
For processing large numbers of samples it is best to split them into batches so that the suggested 10 minutes incubation period is not exceeded. See previous note

NOTE:
The 10 minute incubation may be extended to overnight for convenience. For very rare mRNAs, this may result in a slight increase of signal. However, there is a noticeable decrease in signal for hybridization times between approximately 15 minutes and 10 hours.

C. RNase Digestion of Hybridized Probe and Sample RNA

1. Prepare working dilutions of RNase A/T1 Mix in HybSpeed RNase Digestion Buffer. Standard dilution of RNase A/T1 Mix is 1:100. RNase A/T1 Mix should be vortexed and microfuged briefly before use. Keep at room temperature until used. ***Do not put on ice.***
   RNase T1 (alone) may be substituted for the RNase A/T1 Mix when using a probe from a different species than the sample RNA, or when the target sequence is very AU rich (See Section IV.B. and C. for more information on the use of RNase T1).
2. Add 100 µl of diluted RNase solution (RNase digestion buffer and RNases) to all experimental tubes (***one at a time as they are removed from the 68°C bath vortex and place at 37°C***) and to one tube of each pair of yeast RNA of control tubes. Do not microfuge the samples.
3. Add 100 µl HybSpeed RNase Digestion Buffer (without RNase) to the remaining yeast RNA control tube(s), one for each different probe used. Vortex tubes briefly.
4. Incubate tubes for 30 minutes at 37°C, ***re-vortexing*** samples after 15 minutes.
5. Add 150 µl HybSpeed Inactivation/Precipitation Mix to each tube. Vortex tubes briefly.
   Precipitation of very small fragments (less than 150 bases) can be improved by adding 50 µl of ethanol (for fragments of at least 100 bases) or 100 µl of ethanol (for fragments of at least 50 bases), in addition to 150 µl of HybSpeed Inactivation/Precipitation Mix.
6. Transfer tubes to -20°C freezer for at least 15 minutes. It is not necessary to add additional carrier during this precipitation.

D. Separation and Detection of Protected Fragments

1. Prepare a denaturing polyacrylamide gel suitable for separation of protected fragments of the expected size (typically a 5% polyacrylamide/± 8M urea/ 1 X TBE).
2. Remove tubes from freezer and microfuge for 15 minutes at maximum speed (at least 10,000 x g), preferably at 4°C. Carefully remove all supernatant from each tube. Residual supernatant will cause aberrant migration of bands in gel.
3. Dissolve each pellet in Gel Loading Buffer II by vigorous vortexing and brief microfuging. The volume of gel loading buffer is not critical; resolution of bands is optimal when the gel loading buffer forms a 2-3 mm layer in the well (usually 4-10 µl).
4. Heat all tubes for 3-4 minutes at 90°± 5°C.
5. Re-vortex and re-microfuge tubes briefly.
6. Load samples on polyacrylamide gel and run at approximately 250 volts for about 1 hour in 1X TBE buffer. (See Section VII. for buffer preparation.)
7. If radiolabeled probes were used:
   Transfer gel to filter paper, cover with plastic wrap, and expose to X-ray film room temperature or at -80°C with an intensifying screen.
   The gel can be fixed and dried on a gel dryer. Dried gels preclude diffusion of bands and give sharper resolution on autoradiographs.

If a nonisotopically labeled probe was used:

The gel must be transferred to a positively charged nylon membrane (electroblotting, See Section V.G.). The nonisotopic probe is then detected using an appropriate secondary detection procedure (e.g. Ambion's BioDetect Kit, Cat. #1925)